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Identification of PVK/CAP2b neuropeptides from single neurohemal organs of the stable fly and horn fly via MALDI-TOF/TOF tandem mass spectrometry

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ABSTRACT

MALDI-TOF/TOF tandem mass spectrometry has been applied to determine the complete sequences of the PVK/CAP2b neuropeptides in the stable fly *Stomoxys calcitrans* and horn fly *Haematobia irritans*, insect pests of livestock. This peptidomic analysis of single neurohemal organ preparations allows the unambiguous assignment of internal Leu/Ile positions not distinguishable by previous mass spectrometric techniques. The sequences are as follows: Stoca-PVK/CAP2b-1, AGGASGLYAFPRVa; Stoca-PVK/CAP2b-2, NAKLYPVPRVa; and Haeir-PVK/CAP2b-1, AGGASGLYAFPRVa; Haeir-PVK/CAP2b-1, NAKLYPMPRVa. Both Stoca-PVK/CAP2b-1 and -2 stimulate Malpighian tubule fluid secretion in the stable fly, with EC_{50} values between 3 and 11 nM. The identification of these novel neuropeptides adds to our knowledge of the peptidomes of flies, and can aid in the development of neuropeptide-based control strategies of these insect pests.

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1. Introduction

Neuropeptides are important messenger molecules that occur in a great variety of forms and are implicated in the regulation of critical physiological processes such as diuresis, digestion, development and reproduction [6,7] in insects. In the past several years, new developments in matrix-assisted laser desorption-time-of-flight mass spectrometry (MALDI-TOF MS) have afforded very sensitive de novo sequencing of peptides via direct analysis of single neurosecretory organs or nerves, including those of insects [13,18,19] via the post-source decay (PSD) technique. Alternatively, electrospray ionization (ESI) coupled with tandem MS has resulted in the identification of a number of novel neuropeptides [2,20,22,24,25]. The amount of material needed for ESI-MS experiments is usually larger than the amount necessary for MALDI-MS, since the peptides have to be extracted from the tissue prior to analysis. Both techniques, however, alleviate the need for large numbers

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of specimens and the time-consuming and expensive efforts required to isolate and determine the primary structure of neuropeptides via traditional chromatographic and chemical sequencing techniques [4,8,28]. These MS techniques have also made it possible to make detailed comparisons of the peptide patterns or profiles (i.e., the peptidomes) of closely related insect species [1,3,24]. MS analysis of insect neuropeptides has failed in the past to distinguish between the isomers Leu and Ile, which have identical masses. These earlier studies were limited to low energy fragmentations of the ion of interest. A primary limitation of PSD peptide sequencing is that the internal energies of the $[M + H]^+$ ions are not sufficient to yield the side chain cleavages necessary to distinguish Leu and Ile. However, recent innovations in MALDI-TOF MS have allowed analysis of high-energy collision-induced dissociation of the parent ions of peptides that reveal unique mass patterns for the sidechains of Leu and Ile [11,12]. Indeed, in a recent study we have demonstrated the utility of MALDI-TOF/TOF tandem MS in distinguishing between Leu and Ile in neuropeptides from single neurohemal preparations of insects, specifically PVK/CAP2b (periviscerokinin/cardioacceleratory peptide 2b) sequences from the housefly (*Musca domestica*) and flesh fly (*Neobellieria bullata*) [15].

In this study, we utilize MALDI-TOF/TOF tandem MS to undertake the first identification of the sequences of PVK/CAP2b neuropeptides, including the unambiguous assignment of Leu/Ile positions, from single neurohemal organ preparations of adults of the stable fly *Stomoxys calcitrans* and horn fly *Haematobia irritans*, important livestock pests. PVK/CAP2bs are typical of the abdominal neurohemal system of insects [29], usually stored in abdominal perisympathetic organs. The PVK/CAP2b class of neuropeptides has been shown to elicit both myotropic activity and stimulation of Malpighian tubule fluid secretion in insects (see [29]), physiological processes critical to survival. We report on the diuretic activity of the two PVK/CAP2b sequences native to the stable fly in a stable fly Malpighian tubule fluid secretion bioassay.

2. Materials and methods

2.1. *Insects*

Stable flies (*S. calcitrans*) and horn flies (*H. irritans*) were obtained as pupae from the Knipling-Bushland U.S. Livestock Insects Laboratory in Kerrville, TX. Pupae were kept in cages at 26 °C. Newly emerged stable and horn flies were fed a beef blood meal twice daily for two weeks and provided with sucrose and water ad libitum. Male and female flies <10 days post-emergence were used in all experiments. Stable flies used in fluid secretion assays were obtained as pupae from Professor Mike Lehane (Liverpool School of Tropical Medicine). Adult flies were held at 27 °C and fed a 5% solution of sucrose.

2.2. *Matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS)*

Dissection and sample preparation were performed as previously described [14,18]. MALDI analysis was performed on the ABI 4700 proteomics analyzer (Applied Biosystems, Framingham, MA) [12]. Due to the nature of the samples all acquisitions were taken in manual mode. Initially the instrument was operated in reflectron mode, in order to determine the parent masses. The laser intensity was set just above the threshold required to ionize the neuropeptides. For the tandem MS experiments, the acceleration voltage applied was 1 kV in all cases, and the laser intensity was increased by 10%. The number of laser shots used to obtain a spectrum varied from 500 to 5000, depending on signal quality. In order to change the net amount of collisions to the primary ions in the collision induced dissociation (CID) experiment, the collision cell gas (atmospheric air) pressure was increased. All three gas pressures settings available (none, medium and high) were employed. The instrument was operated in post-source decay mode when no collision gas is used. The true pressure within the collision cell cannot be measured. The fragmentation patterns from these three different settings were used to determine the sequence of the peptide. The fragmentation data obtained in these experiments was handled using the Applied Biosystems Data Explorer® software package.

2.3. *Peptide synthesis*

Stoca-PVK-1/Haeir-PVK-1 (AGGASGLYAFPRVa), Stoca-PVK-2 (NAKLYPVPRVa), and Haeir-PVK-2 (NAKLYPMPRVa) were synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Advanced Chemtech, Louisville, KY) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) under previously described conditions [14]. Crude products were purified on a Waters C_{18} Sep Pak cartridge and a Delta Pak C_{18} reverse-phase column (8 mm × 100 mm, 15 (m particle size, 100 (pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. Delta-Pak C-18 retention times: Stoca-PVK-1/Haeir-PVK-1 (AGGASGLYAFPRVa), 9.0 min; Stoca-PVK-2 (NAKLYPVPRVa), 6.25 min; and Haeir-PVK-2 (NAKLYPMPRVa), 10.95 min. The peptides were further purified on a Waters Protein Pak I125 column (7.8 mm × 300 mm) (Milligen Corp., Milford, MA). Conditions: Flow rate: 2.0 ml/min; isocratic with solvent = 80% acetonitrile made to 0.01% TFA. WatPro retention times: Stoca-PVK-1/Haeir-PVK-1 (AGGASGLYAFPRVa), 9.25 min; Stoca-PVK-2 (NAKLYPVPRVa), 8.75 min; and Haeir-PVK-2 (NAKLYPMPRVa), 9.0 min. Amino acid analysis was carried out under previously reported conditions [14] and used to quantify the peptide and to confirm identity, leading to the following analyses: Stoca-PVK-1/Haeir-PVK-1 (AGGASGLYAFPRVa): A[2.8], F[1.0], G[2.7], L[1.0], P[1.1], R[1.0], S[0.9], V[1.1], Y[1.0]; Stoca-PVK-2 (NAKLYPVPRVa), A[1.0], K[0.9], L[1.0], N[0.9], P[1.9], R[1.0], V[1.9], Y[1.0]; and Haeir-PVK-2 (NAKLYPMPRVa), A[1.0], K[1.0], L[1.0], M[1.0], N[1.0], P[2.0], R[1.0], V[1.0], Y[0.9]. The identity of the peptide analogs was confirmed via MALDI-TOF-MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical, Ltd., Manchester,

UK) with the presence of the following molecular ions (M + H$^+$): Stoca-PVK-1/Haeir-PVK-1 (AGGASGLYAFPRVa), 1266.3 [M + H$^+$]; Stoca-PVK-2 (NAKLYPVPRVa), 1156.6 [M + H$^+$]; and Haeir-PVK-2 (NAKLYPMPRVa), 1188.9 [M + H$^+$].

2.4. *Isolated stable fly Malpighian tubule preparations*

Fluid secretion from isolated stable fly Malpighian tubules was measured using the ''Ramsay assay'' as previously described for housefly tubules [8]. Tubules were removed from 4- to 7-day-old adult flies of both sexes. Flies were dissected under *Musca* saline [8] and both anterior and posterior tubules were transferred to small (10 μl) drops of bathing fluid (a 1:1 mixture of saline and Schneider's *Drosophila* medium) beneath water-saturated liquid paraffin in a Sylgard^{TM} lined Petri dish. The tubules were allowed to equilibrate for 1 h before being challenged with test peptides. Urine escaped from the cut end of the tubule, which was pulled out into the liquid paraffin. Drops of urine were collected at 15 min intervals and their diameter (d) measured as they rested on the Sylgard base of the Petri dish using a Wild digital (MMS235) eyepiece micrometer. Urine volume was calculated as $\pi d^3/6$ and the rate of secretion obtained by dividing the secreted volume by the collection period. Data were normalized by expressing the increase in fluid secretion as a percentage of the response to 10 nM Musdo-K (also native to the stable fly [13]), which was added to all tubules at the end of each experiment. Dose–response curves were prepared using the computer program GraphPad Prism version 4.02 (GraphPad Software, San Diego, CA).

3. Results

3.1. *Determination of PVK/CAP2b sequences in abdominal dorsal sheaths of flies via MALDI-TOF/TOF mass spectrometry*

Direct analysis of abdominal dorsal sheath tissues from the stable fly *S. calcitrans* and horn fly *H. irritans* were conducted via MALDI-TOF/TOF MS. Illustrated in Fig. 1 are the initial MALDI-TOF spectra of the preparations of these two flies that feature the parent ions of the PVK/CAP2b peptides. High energy CID of the PVK/CAP2b peptides reveal unique patterns for the sidechains of Leu and Ile [11,12,15]. As illustrated in Fig. 2, fragments of native Stoca-PVK-2 of the stable fly include a prominent 'w7a' fragment ion at mass 788.3 Da (Fig. 2), indicative of Leu in this peptide. Indeed, the spectrum of the synthetic version of Stoca-PVK-2 containing Leu at position 7 taken under the same conditions was essentially identical. In contrast, if Stoca-PVK-2 contained Ile, the mass spectrum under conditions of high gas would reveal a different mass for the 'w7a' fragment, along with two diagnostic satellite ions, a 'v-ion' and a 'wb-ion'. Thus, Stoca-PVK-2 can be unambiguously assigned the sequence NAKLYPVPRVa (Table 1). Using the same single organ preparations, the sequences of Stoca-PVK-1, Haeir-PVK-1, and Haeir-PVK-2 could also be unambiguously determined under conditions of high gas pressure to be AGGTSGLYAFPRVa, AGGTSGLYAFPRVa, and NAKLYPMPRVa (Table 1), respectively.

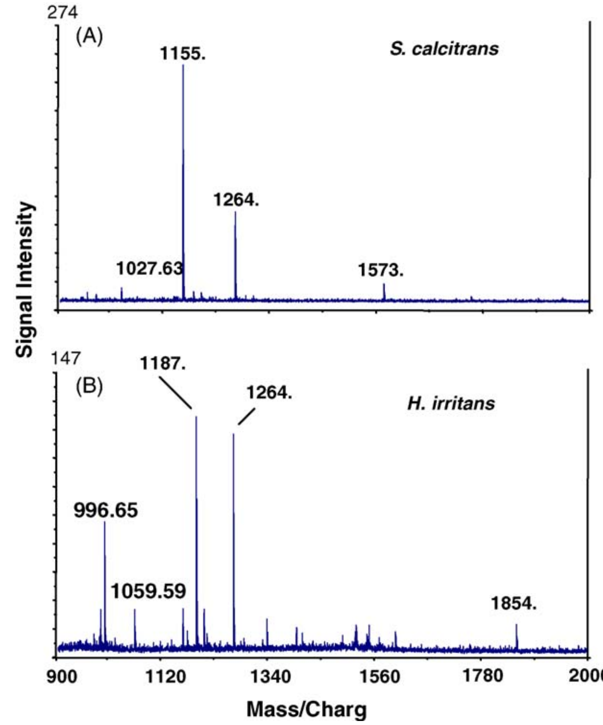

Fig. 1 – MALDI-TOF mass spectra of preparations of the posterior abdominal dorsal sheath of the ventral nerve cord of the stable fly *S. calcitrans* (A) and the horn fly *H. irritans* (B). The dorsal sheath represents a neurohemal release site homologous to perisympathetic organs which become incorporated into the dorsal ganglionic sheath during the metamorphosis of cycloraphous flies. The MH+ values are as follows: for Stoca-PVK/CAP2b-1 (1264.70), Stoca-PVK/CAP2b-2 (1155.72), Heair-PVK/CAP2b-1 (1264.68), and Heair-PVK/CAP2b-2 (1187.67).

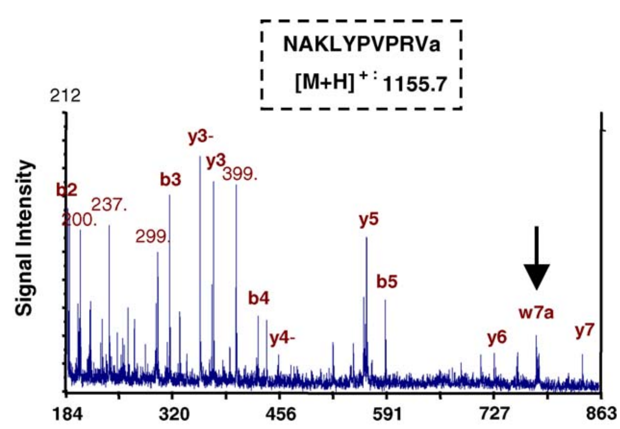

Fig. 2 – A MALDI-TOF/TOF tandem mass spectrum of native Stoca-PVK-2 under conditions of high gas pressure (arrow identifies the 'w7a' fragment ion at 788.3). The mass spectra of the native and synthetic Leu variant are essentially identical.

Table 1 – Amino acid sequences of PVK/CAP2b peptides native to the stable fly (*S. calcitrans*) and horn fly (*H. irritans*) determined by MALDI-TOF-TOF tandem mass spectrometry compared with previously determined sequences from other flies

Species	PVK/CAP2b-1	PVK/CAP2b-2
S. calcitrans	AGGASGLYAFPRVa	NAKLYPVPRVa
H. irritans	AGGASGLYAFPRVa	NAKLYPMPRVa
M. domestica	AGGTSGLYAFPRVa	ASLFNAPRVa [15]
N. bullata	NGGTSGLFAFPRVa	AGLIVYPR[L/I]a[a] [15]
D. melanogaster	GANMGLYAFPRVa	ASGLVAFPRVa [27]
An. gambiae	GPTVGLFAFPRVa	QGLVPFPRVa [17]

[a] MALDI-TOF/TOF tandem MS cannot distinguish between Leu and Ile at a C-terminal position [14].

3.2. *Fluid secretion activity of PVK/CAP2b sequences on stable fly Malpighian tubules*

Synthetic replicates of the two PVK/CAP2b sequences native to the stable fly were evaluated for diuretic activity on isolated Malpighian tubules. As can be seen in Fig. 3 and Table 2, Stoca-PVK-1 and Stoca-PVK-2 stimulated stable fly Malpighian tubule fluid secretion with observed EC_{50} values of 3.4 and 11.0 nM, respectively (Table 2). The peak response to both peptides was ~25% of that obtained after the addition of 10 nM Musdo-K (also native to the stable fly [13]), and represented an 80% increase over the basal rate of fluid secretion.

4. Discussion

PVK/CAP2b peptides have been found in the stable fly *S. calcitrans* and horn fly *H. irritans* via MALDI-TOF MS analysis of preparations of neurohemal tissues [24]. PVK/CAP2b peptides of insects are typical of neurosecretory neurons in the abdominal ganglia and are accumulated in perisympathetic organs until release. Larval perisympathetic organs of cyclorraphous Diptera, however, become incorporated into the dorsal ganglionic sheath [16] during the metamorphosis. The abdominal dorsal sheath, which was dissected in this study, therefore contains relatively large amounts of peptidergic neurohormones. The molecular ions and incomplete sequences observed in the MALDI-TOF/TOF mass spectrometric studies operated in reflectron mode (Fig. 1) on the stable fly and horn fly were, Stoca-PVK-1 (AGGASG[L/I]YAFPRVa; *m/z* 1264.70) and Stoca-PVK-2 (NAK[L/I]YPVPRVa; *m/z* 1155.72); and Haeir-PVK-1 (AGGASG[L/I]YAFPRVa; *m/z* 1264.68) and Haeir-PVK-2 (NAK[L/I]YPMPRVa; *m/z* 1187.67). When the peptides were fragmented under conditions of high collision energy, the collision-induced fragments reveal distinct side-chain fragmentations. Stoca-PVK-2 demonstrated a fragmentation pattern indicative of a Leu in position 7 (Fig. 2), and this same fragmentation pattern was observed in mass spectra of a synthetic replicate containing Leu. Thus, Stoca-PVK-1 can be unambiguously assigned the sequence NAKLYPVPRVa (Table 1). Using the single organ preparations, the sequences of Stoca-PVK-1, Haeir-PVK-1 and Haeir-PVK-2 could also be unambiguously determined under conditions of high collision energy to be AGGASGLYAFPRVa, AGGASGLYAFPRVa and NAKLYPMPRVa (Table 1), respectively. Thus, the PVK/CAP2b-1 sequences of the stable fly and horn fly are identical, and the PVK/CAP2b-2 sequences differ only by a single residue in the fourth position from the C-terminus (V versus M).

It is clear that the Leu at the position located seven residues from the C-terminus in these four PVK/CAP2b sequences from stable fly and horn fly is conserved within and across species (Table 1). Leu at this specific position is typical of other PVK/CAP2b of insects that were sequenced by Edman degradation in earlier studies [5,9,20,21,23] or for which genes have been published already [10,26,27]. In general, PVK/CAP2b sequences are well conserved throughout the six species of flies that have been studied to date (Table 1).

Both the stable fly peptides stimulated Malpighian tubule fluid secretion, with Stoca-PVK/CAP2b-1 being slightly more potent than Stoca-PVK/CAP2b-2, although the difference was not significant. The maximum response was equivalent to an 80% increase in the basal rate of secretion, which is consistent with data for other dipteran insects [17], and is about 25% of the response of stable fly tubules to 10 nM Musdo-K.

In summary, MALDI-TOF/TOF tandem MS has been used to identify the sequences for the PVK/CAP2b neuropeptides in the stable fly and horn fly via direct analysis of nerve tissue. This analysis includes an unambiguous assignment of the monoisotopic residues Leu versus Ile. The work adds to our knowledge of the peptidomes of flies; and the identification of

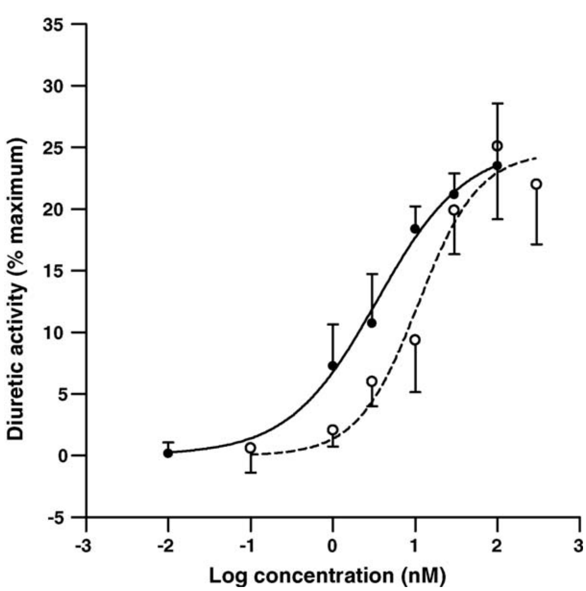

Fig. 3 – Dose–response curves for Stoca-PVK/CAP2b-1 (solid line and symbols) and -2 (dashed line and open symbols). Data are expressed as the mean ± S.E.M for six replicates.

Table 2 – Fluid secretion activity of native Stomoxys-PVK/CAP2b-1 and -2 on stable fly (*S. calcitrans*) Malpighian tubules

Peptide	Sequence	Fluid secretion[a] $[EC_{50}]$ (nM)
Stoca-PVK/CAP2b-1	AGGASGLYAFPRVa	3.4 (0.6–18.9)
Stoca-PVK/CAP2b-2	NAKLYPVPRVa	11.0 (3.8–31.6)

[a] Values in parentheses, represent the 95% confidence limit (CL).

the specific structures of the PVK/CAP2b neuropeptides, implicated in the regulation of diuretic and myotropic processes, may aid in the development of mimetic analogs capable of disrupting these critical physiological processes in pest flies.

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